American Fern Journal 100(3):159–166 (2010)

Recovery of Viable Germplasm from Herbarium Specimens of *Osmunda regalis* L.

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ABSTRACT.—The conservation of pteridophytes presents a demanding challenge because many species in the world are thought to be threatened with extinction, as they are very sensitive to environmental disturbance. *Ex situ* actions provide an important conservation strategy, so the Germplasm Bank of Tuscia Botanic Garden, with the Herbarium UTV (Tuscia University, Viterbo–Italy), has undertaken a project for the conservation of threatened pteridophytes of the Italian flora, like *Osmunda regalis*, the Royal Fern, a species with chlorophyllous spores that is declining in Italy because it is linked to vulnerable habitats. As a part of the project, this work presents first results of *in vitro* reproduction of the Royal Fern using spores from *exsiccata* (UTV samples collected and dried in 1989 and 2001). Our results also highlight the value of herbarium specimens in biodiversity conservation, providing a useful method to reproduce species that are threatened or extinct in the wild, at least at a local level, so as to plan eventual reintroductions using the native germplasm.

KEY WORDS.—*Osmunda regalis*, chlorophyllous spores, herbarium, *in vitro* reproduction, biodiversity conservation

The conservation of pteridophytes presents a demanding challenge, but this highly diverse group is underrepresented on the global Red List. The International Union for Conservation of Nature (IUCN) listed 770 species of pteridophytes world-wide as threatened (Walter and Gillett, 1998), but only 211 in the 2008 Red List (http://www.iucnredlist.org) due to difficulty in assessing species with the new criteria because of significant gaps in the knowledge of their biology, distribution and conservation status (Chandra *et al.*, 2008). Although the species evaluated by IUCN only represent 1.6% of the global described species (13,025) (Arcand and Ranker, 2008), over 10% of the species in the world are thought to be threatened with extinction (Jermy, 1990) and many others are declining as they are very sensitive to environmental perturbations. Moreover, a tendency to be concentrated in endangered regions and habitats, and frequent local endemism, especially on islands, means that a significant proportion of species may be at risk (Given, 2002).

Successful *in situ* conservation of ferns and their-allies within their natural habitats should always be the primary goal of their conservation. However, when this is not possible, additional methods exist to supplement and complement the *in situ* actions, and they are needed to increase the chances of survival of individual species (Page *et al.*, 1992). *Ex situ* approaches provide an

important conservation strategy (Pence, 2002; 2008), but methodologies for the long-term preservation in germplasm banks are not well established.

Pteridophyte spore viability depends on spore type, chlorophyllous and non-chlorophyllous, or taxonomic group (Lloyd and Klekowski, 1970). Biochemical and metabolic factors, depletion of respiratory substrate, loss of membrane integrity, inactivation of enzymes and growth promoters, and chromosomal aberrations or other genetic mutations are cited as possible causes of the rapid loss of viability during storage of non-green spores (Page *et al.*, 1992; Beri and Bir, 1993). In green spores, the high respiratory rate or the failure to recover photosynthetic activity after desiccation are suggested to cause the rapid loss of viability (Jones and Hook, 1970; Lloyd and Klekowski, 1970). There is a narrow window of water contents appropriate for storage at conventional temperatures, so Ballesteros and Walters (2007a, b) have proposed that damage by desiccation or cooling can be prevented by precise control of the water content within spores.

Lloyd and Klekowski (1970) have made comparisons between the viability of chlorophyllous and non-chlorophyllous spores of several species of ferns; they observed that spores with chloroplasts germinate more rapidly than spores without chloroplasts (within one or two days after sowing), but this ability to germinate declines rapidly with their age. Moreover their results show that, under laboratory conditions (room temperature), green spores die early, averaging a life-span of only 48 days, while non-green spores live on average up to 2.8 years. Lebkuecher (1997) suggested that the extremely short viability of chlorophyllous spores of *Equisetum hyemale* L. may result from the inability to recover the photosynthetic competence when the spores were re-wetted after desiccation. Usually, the viability of chlorophyllous spores can be measured in days and weeks rather than in months and years (Lloyd and Klekowski, 1970).

Factors affecting viability of spores (green and non-green) are rather clear, but little is known about the factors affecting spore longevity under herbarium conditions. There are several reports of viability of seeds after lengthy periods of storage in herbaria (Freeman, 1983; Hill, 1983; Willan, 1987; Bowles *et al.*, 1993; Moreno Casasola, 1996), but there are only a few studies concerning pteridophytes. These reports indicate that the non-chlorophyllous spores from herbarium collections of some fern species remain viable for decades. Jonhson (1985) reported that the spores of certain *Marsilea* species were capable of germination after 99–100 years and Windham *et al.* (1986) provided germination data of 50-years-old spores of *Pellaea truncata* Goodding. This kind of longevity is quite common for species with non-chlorophyllous spores that live in xeric habitats, while mesophilic species, such as *Cystopteris protrusa* (Weath.) Blasdell e *Woodsia obtusa* (Spr.) Torrey, lose the capability to germinate after a few years (Windham and Haufler, 1986).

For herbarium specimens, the most important factor controlling spore longevity, aside from the biologic features of the ferns, seems to be the type of treatment used to reduce insect infestations. Viability declines rapidly in herbarium specimens treated with chemical methods and heat, while exposure

to low temperatures seems to have few negative effects on species with non-chlorophyllous spores (Windham and Haufler, 1986).

The Germplasm Bank of the Botanic Garden of Tuscia with the Herbarium UTV (Tuscia University, Viterbo - Italy) has undertaken a project for *ex situ* conservation of threatened pteridophytes of the Italian flora, in order to collect and preserve spores both from natural populations and from *exsiccata* (Magrini *et al.*, 2006). As part of this project, the present work aims to reproduce *Osmunda regalis* L. *in vitro* starting from spores taken from Herbarium UTV *exsiccata*. The distribution area of the Royal Fern includes the warm and temperate regions of Europe, Asia, Africa, and America; in Italy is fairly common in Tuscany and Sardinia, while it is very rare or threatened in most Italian administrative regions (Conti *et al.*, 1997), and no longer found in four Italian regions (Conti *et al.*, 2005). The choice of this subcosmopolitan species with chlorophyllous spores derives from the observation of its increasing decline in Italy, especially considering the fragmentation and isolation of its populations in Central Italy (Landi and Angiolini, 2007). We can assume that its survival is strongly dependent upon the availability of suitable environmental conditions in vulnerable habitats such as bogs, marshes, swamps, streams, and moist forests. Therefore, increasing *ex situ* conservation strategies for the Royal Fern, like cultivation in botanic gardens and long-term storage of spores in germplasm banks, is needed for eventual re-introductions in those sites where it is already disappeared or it is threatened. Since we are not aware of published data on reproduction from *exsiccata* of species with chlorophyllous spores, we present here the first results of our study aimed to reproduce *Osmunda regalis in vitro* from spores taken from Herbarium UTV specimens, and show the recovery of viable green spores from *exsiccata* after long periods of conservation.

Materials and Methods

Among the material of the Herbarium UTV, two *exsiccata* collected from two localities within the National Park of the Tuscan Archipelago were selected. Spores of *Osmunda regalis* were collected from the *exsiccata* UTV 15510 of 05/24/1989 (17 years of storage) from Giglio Island (Grosseto - Tuscany), and UTV 16791 of 05/17/2001 (five years of storage) from M. Capanne, Elba Island (Livorno - Tuscany). The spores for the control tests were collected from a wild population (summer 2006) in a gorge near a ferruginous water stream within the Monterano Regional Nature Reserve (Rome – Latium), and they did not undergo any treatment, nor dehydration or freezing.

In UTV all incoming *exsiccata* underwent two disinfestation treatments, each a month apart: at −35°C for 3 days, thawed for 24 hours and frozen again for 3 days (4 cycles of freezing and thawing). Then, the herbarium specimens were stored in closed polyethylene bags with an internal relative humidity equal to 52% ± 2.

All spores were measured using an ocular micrometer to test their maturity, and were then soaked in distilled water for 24 h. After, they were rinsed three

times with sterile distilled water and centrifuged at 6000 rpm for 3 min between rinses (Fernandez *et al.*, 1997; 1999; Fernandez and Revilla, 2003). The spores (1,000 collected from UTV 15510, and 2,000 from UTV 16791) were sown in sterile plastic Petri dishes (6 cm diameter) containing 10 ml of Knop medium (Knop, 1865) with a Nystatin solution (100 U/ml^{-1}) added as a fungicide (Quintanilla *et al.*, 2002). The medium was adjusted to pH 5.8, and supplemented with 0.7% agar (plant tissue culture grade). The dishes were sealed with Parafilm to reduce contamination and to prevent excessive water loss (Morini, 2000). The cultures were maintained under cool-white fluorescent illumination (Osram Dulux L 36W/840 Lumilux, 2900 lm), a 12-h photoperiod (Dyer, 1979; Windham *et al.*, 1986), and a temperature of 20 ± 2°C (Dyer, 1979; Sheffield *et al.*, 2001; Quintanilla *et al.*, 2002). The dishes were examined microscopically for spore germination (defined as the first emergence of the rhizoid) and morphological studies. After germination, young gametophytes were transferred into the same medium freshly prepared, and sterile distilled water was added to enhance fertilization (Raghavan, 1989); mature gametophytes with the first sporophytes were then transferred into sterilized soil.

Results

Compared to the control germination percentage (about 100%), germination of spores collected from *exsiccata* was low (<1% for UTV 15510, 2% for UTV 16791). Moreover, we observed that spores from *exsiccata* have a longer time to germination when compared to the control, as shown in Figure 1: spores of 2001 germinated in 20 days from sowing, spores from samples of 1989 required over 30 days, whereas fresh spores germinated after 2–4 days. For the spores of 2001, we observed the subsequent phases of the reproductive cycle, until the development of the sporophytes (Fig. 2), while the germinated spores of 1989 died shortly after germination. Despite the long germination times, the development times of the gametophyte and of the sporophyte did not show significant differences compared to the control; we observed the cordate gametophytes after 35–40 days from germination, and the sexually mature ones after 60–70 days from germination. Microscopic observations (stereomicroscope and SEM) made it possible to verify the integrity and the proper development of the prothallia, and the presence of archegonia and antheridia. The first sporophytes were observed after 200–210 days after germination, but the development of the others was distributed over the next 35–60 days (Fig. 2).

Discussion

We report the first results of *in vitro* reproduction of *Osmunda regalis* using spores taken from *exsiccata*, providing new information on the viability of chlorophyllous spores, and useful data to further research on the best conditions and procedures for long-term conservation in germplasm banks. The germination of 17 and five year old chlorophyllous spores provides valuable information, especially when compared with previous studies

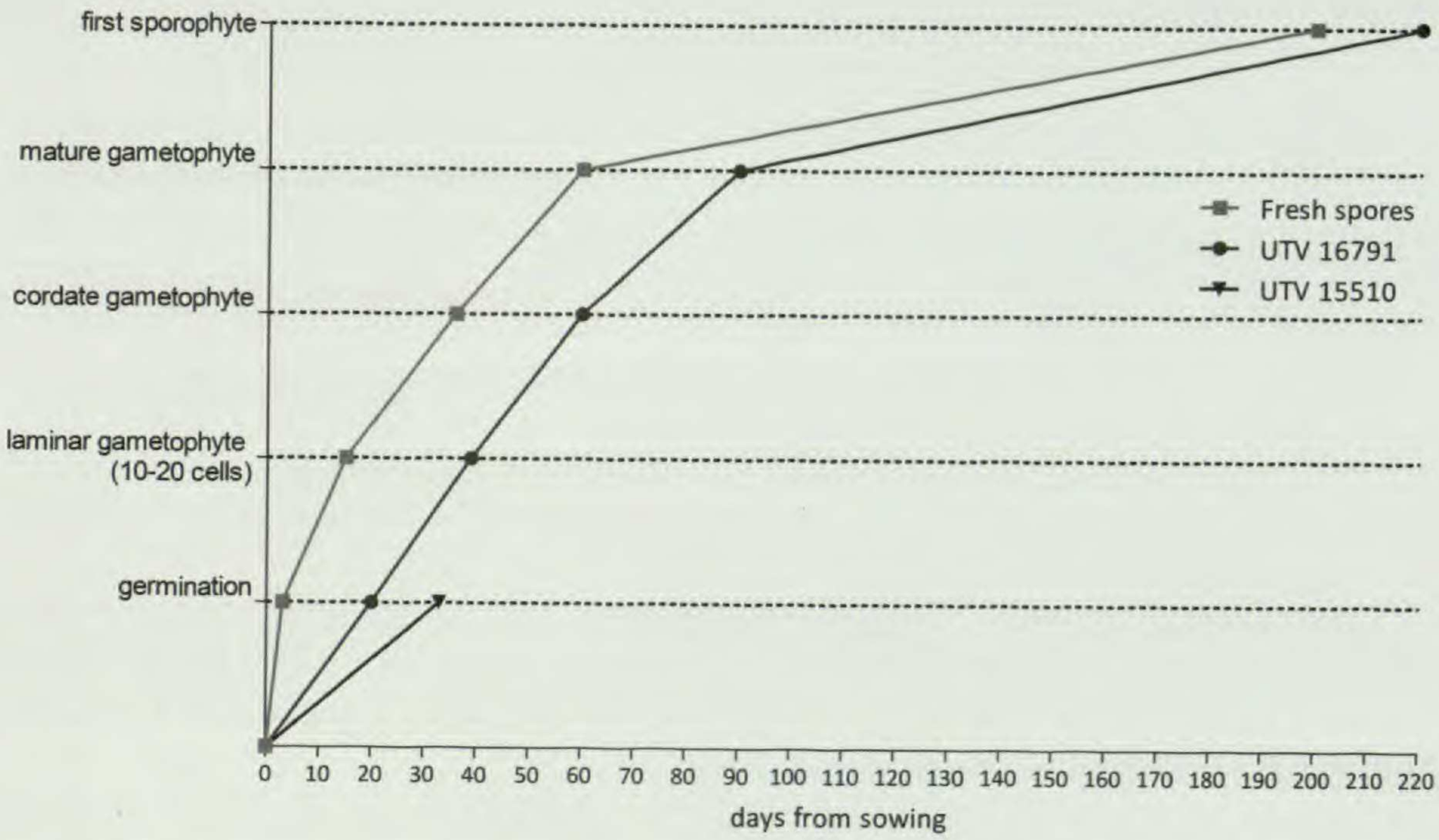

FIG. 1. Trend analysis of the *in vitro* reproduction of *Osmunda regalis*: the time of germination and subsequent development phases of the reproductive cycle.

(Lagerberg, 1908; Gerhardt, 1927; Okada, 1929; Lloyd and Klekowski, 1970) that provide germination data after a maximum of 150 days of storage at room temperature. Spore germination and subsequent sporophyte development from the five year old *exsiccata* could not be the product of cross contamination from newer *exsiccata* placed in the herbarium, because the specimen UTV 16791 of 2001 was the most recent specimen that had fertile fronds.

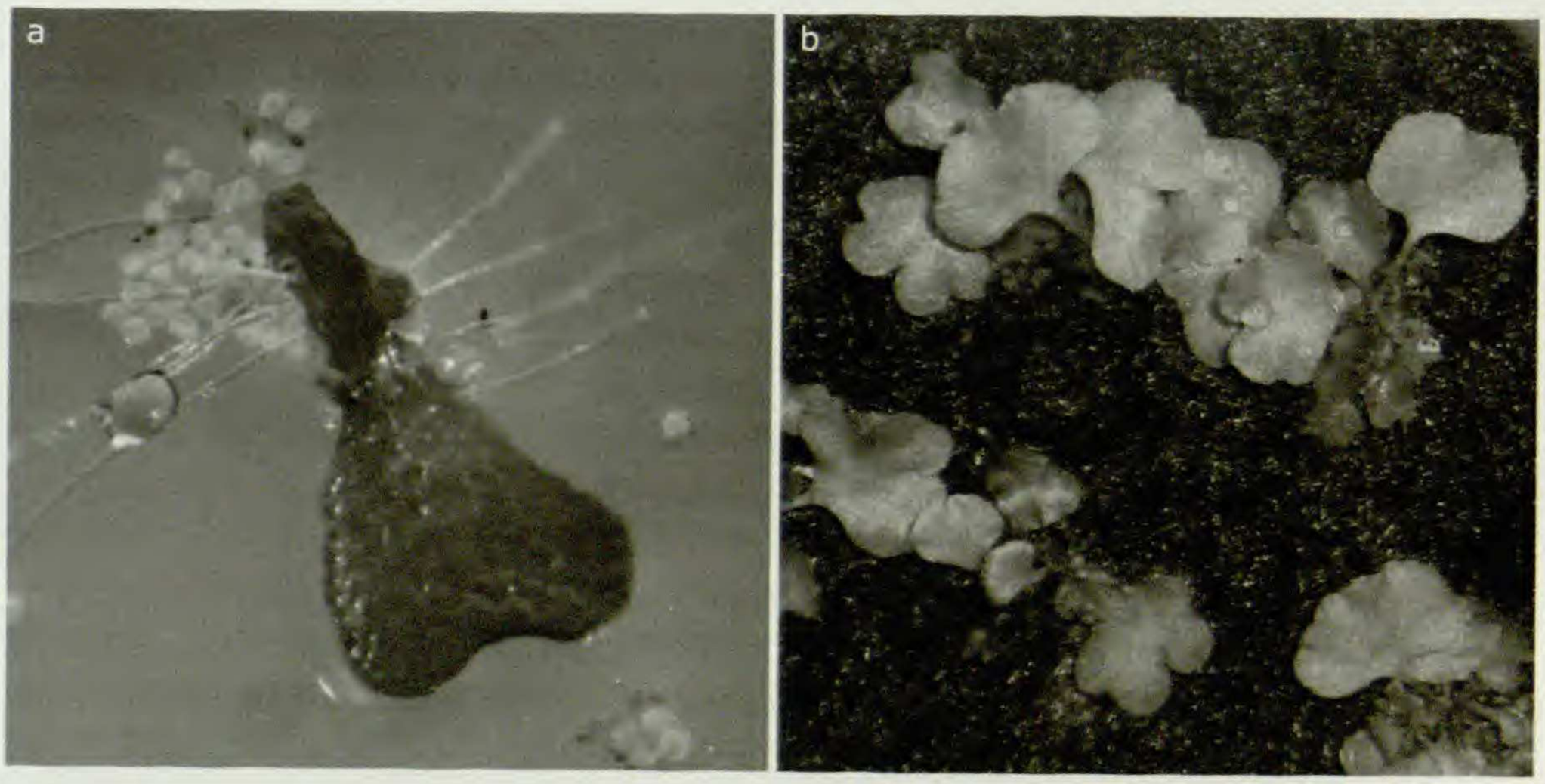

FIG. 2. **(a)** Young gametophyte with apical notch and **(b)** first leaves of the sporophytes obtained by spores collected from the *exsiccatum* UTV 16791 of 05/17/2001 (5 years old).

The viability of the spores of the *Osmundaceae* is considered to be of short duration. Lagerberg (1908) showed that the spores of *Osmunda regalis*, stored at room temperature, may be viable for two months, and Okada (1929) reported that the spores of certain *Osmunda* species are viable for a period of days (*Osmunda cinnamomea* L. for 43–54 days and *Osmunda japonica* Thunb. for 23–43 days). Furthermore, Gerhardt (1927) found that germination time increased with the age of the spores: spores three days old have germinated in one day; 34 days old in four days; 130 days old in seven days; 150 days old in 20 days; and spores 225 days old did not germinate. Our data confirm that the time necessary for germination is directly correlated with the age of spores (Lloyd and Klekowski, 1970), but that the development times of gametophytes and sporophytes do not show any correlation with spore age.

These results provide additional value to the herbarium *exsiccata* for conservation purposes. Herbaria are repositories not only of dried specimens for morphological or taxonomical studies, but are also a source of DNA for phylogenetic research (Rogers and Bendich, 1985; Cano and Poinar, 1993; Savolainen *et al.*, 1995; Cozzolino *et al.*, 2007), and even of living germplasm which have significant potential in the recovery of rare or endangered plant species, including lost genotypes, self-incompatible species, or those subject to inbreeding depression. Use of herbarium spores may be critical for restoring reproduction and/or genetic diversity in small or restored populations (Bowles *et al.*, 1993). This can be a useful method to reproduce species that are threatened or extinct in the wild, at least at a local level, so as to plan the re-introductions using the native germplasm (Lledó *et al.*, 1996; RENPA, 2007).

Herbaria are nonrenewable resources of germplasm of extinct or endangered species that could be appointed to special storage programs which would help to maximize longevity of spores using freezing treatments, but avoiding curatorial techniques like microwaving, excessive heat treatment or pesticides that cause abrupt loss of germplasm viability along with potential changes in plant morphology (Hill, 1983; Bacci *et al.*, 1985; Windham *et al.*, 1986). The spores taken from *exsiccata* and used for our tests were dehydrated and treated with four cycles of freezing and thawing, that are the conventional disinfestation treatments in UTV, so exposure to low temperatures seems to have little effect on spore viability.

We are continuing research on the development of tests with spores from various herbaria (CAG, FI, SIENA), selected from *exsiccata* with different age, chosen at regular intervals, to confirm the correlation between age and longevity of chlorophyllous spores under the same disinfestation treatments.

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